Masking selected sequence variation by incorporating mismatches into melting analysis probes

Rebecca L Margraf*+, Rong Mao*†, W Edward Highsmith^,

Leonard M Holtegaard^, Carl T Wittwer*†

*ARUP Institute for Clinical and Experimental Pathology,

500 Chipeta Way, Salt Lake City, UT 84108, USA

†Department of Pathology, University of Utah Medical School,

Salt Lake City, UT 84132, USA.

^ Molecular Genetics Laboratory, Mayo Clinic, Rochester, MN, 55902, USA.

+ Corresponding author. Mailing address: Advanced Technology Group, ARUP, 500 Chipeta Way, Salt Lake City, UT 84108. Phone: (801) 583-2787. Fax: (801) 584-5114. E-mail: rebecca.margraf@aruplab.com.

Running title: Masking sequence variation in probe melting analysis

T_m, Melting temperature; MEN2, multiple endocrine neoplasia type 2; FMTC, familial medullary thyroid carcinoma; HPA, human platelet antigen; SNP, single nucleotide polymorphism.

Abstract:

Hybridization probe melting analysis can be complicated by the presence of sequence variations (benign polymorphisms or other mutations) near the targeted mutation. We investigated the use of 'masking' probes to differentiate alleles with similar probe melting temperatures. Selected sequence variation was masked by incorporating deletions, unmatched nucleotides, or universal bases into hybridization probes. Such masking probes create a probe:target mismatch with all possible alleles at the selected polymorphic location. Any allele with additional variation at another site is identified by a lower probe melting temperature than alleles that vary only at the masked position. This technique was applied to RET and HPA6 mutation detection using unlabeled hybridization probes, a saturating dsDNA dye and high-resolution melting analysis. Masking probes identified all targeted mutations when at least one base pair separated the mutation from the masked variation. Polymorphisms immediately adjacent to mutations could usually be masked, except in certain cases, such as with single base deletion probes when both adjacent positions have the same polymorphic nucleotides. The masking probes can also localize mutations to specific codons or nucleotide positions. Masking probes can simplify melting analysis of complex regions and can eliminate the need for sequencing.

Key words: Polymorphism, mutation, probe hybridization, high-resolution melting analysis, masking and RET.

Introduction

There are many methods for identifying point mutations, including gel analysis after restriction enzyme digestion, allele-specific PCR, microarrays, sequencing and probe-based methods that generate allele-specific melting curves (1-7). The latter method uses hybridization probes, often of wild type sequence, to detect any sequence alteration under the probe by melting temperature (T_m). Mutation detection by hybridization probe melting analysis is a rapid, closed-tube assay that does not require any post-PCR sample processing. However, the need for expensive fluorescently-labeled probes is a disadvantage of this method.

High-resolution melting of PCR products in the presence of saturating dsDNA binding dyes can detect and genotype most sequence variation by differences in the shape and position of the melting curve (8-10). However, in clinical diagnostic assays, it is commonly believed that the extra specificity of a probe is necessary. In order to provide probe specificity without the cost of fluorescently-labeled probes, an unlabeled probe method was recently introduced (11). In this method, three oligonucleotides are required, two PCR primers and one internal probe which is 3' phosphorylated to prevent extension. The melting transition of the unlabeled probe is monitored by the fluorescence of LCGreen® PLUS dye as the probe:target duplex melts.

When wild type hybridization probes are used for genotyping, melting analysis does not distinguish all possible sequence variants (11-14). Sequence variation under the detection probe can include benign polymorphisms, variants of undetermined significance and pathogenic mutations. Sometimes polymorphisms can be eliminated from analysis by changing the size or location of the probe. In other cases this may not be possible, depending on the position and number of polymorphisms relative to the targeted mutation. For example, the wild type hybridization probe used to detect the prothrombin 20210 mutation could not differentiate the

sequence variants of 20209 C>T and 20221 C>T using standard hybridization probe analysis (12). Thermodynamic predictions suggests that 32% of all single base mismatches with wild type probes have a T_m within +/- 1°C of a targeted mutation (15). Because unique alleles can have nearly the same T_m with detection probes (11-16), there is a risk of interpreting polymorphism as mutations or mutations as polymorphisms. The presence of polymorphisms near targeted mutations is possible genome wide, as about 90% of human sequence variation is due to single nucleotide polymorphisms (SNPs), with an average of six SNPs per gene coding sequence (17,18).

This report systematically tests techniques for masking non-targeted sequence variation in melting curve analysis using hybridization probes. Probes were designed with a 'mask' over the non-targeted sequence variation, which creates an artificial mismatch with all possible alleles. Wild type and masked variant alleles have a single mismatch with the probe and a similar T_m. In contrast, the targeted mutation results in an additional mismatch with the probe and a lower T_m than the wild type and masked alleles. This report demonstrates the use of three types of masking probes: probes with deletions (one to three base pairs), probes that incorporate a universal base, and probes with an unmatched nucleotide that does not complement the possible nucleotides at the masked, polymorphic location. The masking technique was demonstrated with the RET proto-oncogene and human platelet antigen type 6 (HPA6), where polymorphisms are near or immediately adjacent to targeted mutations (19-22). This technique was also used to identify the location of sequence variation under the probe.

Materials and Methods

Samples: De-identified genomic DNA from MEN2/FMTC patients of known RET mutations or normal controls from the Mayo Clinic followed IRB protocol **701-04**. All RET genotypes were confirmed by sequence analysis and compared to the RET genomic sequence in GenBank AJ243297 (sequencing of the generated PCR products with BigDye terminator chemistry from Applied Biosystems, Foster City, CA).

All the RET sequence variations tested were single nucleotide polymorphisms (SNPs). RET SNPs that alter RET function to cause MEN2 syndromes are mutations and benign SNPs are polymorphisms, according to conventional terminology (19,23). All variant RET samples were heterozygous for mutations or polymorphisms unless otherwise stated. RET sequence variation is listed by the codon number, wild type codon sequence, and the variant sequence with the mutant nucleotide in bold (e.g. 618 (TGC>T**A**C)), while a nucleotide resulting in a benign polymorphism is underlined. RET exon 13 has two reported mutations at codon 768 (GAG>GA**C** or GA**T**) and a common polymorphism at codon 769 (CTT>CTG) (19,20,22). RET exon 10 and 11 have mutations at codons 609, 611, 618, 620, 630, and 634, which include all possible nucleotide changes from the wild type codon (TGC) for the amino acid cysteine (Table 1) (20,22). Exon 11 has a polymorphism at 631 (GAC>GAT).

The HPA6 targeted mutation is at codon 489 (CGG>C**A**G) which is immediately adjacent to the polymorphic sequence at codon 489 (CGG>CGG, CGA, or CGC) with 63%, 37%, and <1% allelic frequencies, respectively ((21) and Genbank M57489). Homozygous HPA6 templates were engineered from de-identified patient genomic DNA (24) and included all possible combinations of wild type, mutant and neighboring polymorphic sequences. All artificial templates were sequenced to confirm genotype.

Primers and probes: All primers and probes were synthesized at Integrated DNA Technology (IDT, Coralville, IA). The primer locations for the different RET exons were chosen to create amplicons that included all known pathogenic mutations and, if possible, to exclude any polymorphisms from analysis (19,20,22). Primers were designed using Primer 3 software (25).

The probes were 3' phosphorylated to prevent extension during PCR. The universal base probes contained a 5'-nitroindole at the masking site. Primer and wild type probe sequences are listed in Table 1. The masking probe type, masking position and the location of the polymorphic sequences are diagrammed in each of the figures.

PCR: Sample DNA was amplified by asymmetric PCR using the LightCycler FastStart DNA Master Hybridization Probe Kit (Roche Diagnostics Corp, Indianapolis, IN) and 1 μL of purified DNA (50-100 ng) with a final reaction volume of 10 μL. PCR included 1x FastStart master hybridization mix, 2 mM of $MgCl_2$, 0.5 μM excess primer, 0.05 μM limiting primer, 0.01 U/μL Uracil-DNA glycosylase (Roche Molecular, Indianapolis, IN), 0.5 μM unlabeled probe and 1x LCGreen PLUS (Idaho Technology, Salt Lake City, UT). The reverse primer was in excess for exons 10 and 13, while the forward primer was in excess for exon 11.

Thermocycling for the RET exons was performed on a LightCycler® (Roche) and included a uracil-DNA glycosylase step at 50°C for 10 minutes, polymerase activation at 95°C for 10 minutes and sixty PCR cycles consisting of denaturation at 95°C for 1 second, annealing at 62°C for 1 second and extension at 72°C for 10 seconds. HPA6 was amplified with the same protocol, except that a 68°C annealing temperature was used for 55 PCR cycles with a 9:1 (forward to reverse) asymmetric primer ratio. After PCR, unlabeled probes were hybridized to the target ssDNA by heating to 95°C followed by rapidly cooling the samples to 40°C.

High-resolution melting. Analysis was performed on a high-resolution melting instrument, the HR-1™ (Idaho Technology, Salt Lake City, UT). The LightCycler capillaries were transferred into the HR-1 and heated at 0.3°C/sec. Melting data for RET exons was acquired between 55°C and 95°C and HPA6 between 65°C and 95°C. The melting data was directly converted to a derivative plot (-dF/dT vs. temperature) with the HR-1 software. The highest -dF/dT value of the derivative melting peak was used as the melting temperature (T_m). The T_m difference (ΔT_m) is the difference in T_m between the wild type allele and the variant allele (26).

Results

Masking polymorphisms near targeted mutations:

RET exon 13 has a common benign polymorphism of 0.26 allelic frequency separated by two base pairs from a rare pathogenic mutation (Figure 1A) (19,20,22). Both the polymorphism and the targeted mutation have one mismatch with the wild type probe, which resulted in similar probe melting temperatures (Figure 1B). Masking probes that incorporated a universal base, deletion, or an unmatched nucleotide over the non-targeted polymorphism location were evaluated for RET exon 13 (Figure 1C-F). The unmatched nucleotides used for the masking probes did not complement the possible nucleotides at the polymorphism location (A>C). Each masking probe reduced all possible alleles to one mismatch status with the probe, creating a nearly identical T_m for the masked polymorphism allele as for the wild type allele. The targeted mutant allele had an additional mismatch with the masking probes and was clearly distinguished by a 3°C lower T_m than the wild type or masked polymorphism alleles.

RET exon 11 has two codons of possible pathogenic mutations at 630 and 634, and a polymorphism at codon 631. In order to analyze the two pathogenic codons but not the codon 631 polymorphism, a masking deletion and two unmatched nucleotide probes were tested (Figure 2). With all three probes, the codon 631 polymorphism was masked from analysis with a nearly identical T_m as the wild type allele, allowing the lower T_m mutant alleles to be clearly detected.

Locating the position of sequence variation under probes:

Masking probes can locate the position of a mutation to a particular sequence location. RET exon 10 mutations are mainly restricted to four pathogenic codon locations: 609, 611, 618, and 620 (19,20,22). Each wild type probe over two of the pathogenic codons can detect a mutation, but does not identify which codon contains the mutation (Figure 3B and 3E). Figure 3 demonstrates how the codon of exon 10 mutations can be located. Masking probes were designed with a three base pair deletion over one of the codons. An allele with a mutation within the masked codon will have the same T_m as the wild type allele. However, any mutation outside of the masked location will have an additional mismatch with the deletion probe and result in a lower T_m. In each case, mutations within the masked codons were as stable as the wild type allele, whereas alleles with the mutation outside of the masked codon were clearly identified by lower T_ms.

Masking sequence variation immediately adjacent to the targeted mutation:

The positional effects of single base masking deletions in the probe relative to targeted mutations are shown in Figure 4. Five different single base deletion probes were designed across RET exon 11 codon 634 (TGC). Alleles with mutations in the second position of codon 634

(dark blue) had a T_m 3-4°C below the wild type allele with all probes (Figure 4B-E, G), except with one of the deletion probes (Probe 4, Figure 4F). This probe deletion was over the mutation site, which masked all the codon 634 second position mutations and resulted in a similar allelic T_ms as the wild type allele. Similarly, a mutation at the third position of codon 634 (light blue, TGC>TG**G**) had a T_m 2-3°C below the wild type allele with all probes (Figure 4B-F), except when the deletion was over the mutation site, masking only this mutation (Figure 4G). Mutations at the first position of codon 634 (red) were also masked by probes with a deletion over the mutation site (Figure 4E). However in this case, when the deleted base was immediately adjacent to the mutation (Figures 4D and 4F), the T_ms of the mutations were very similar (within 0.8°C) to the wild type T_m.

The HPA6 G>A mutation is immediately adjacent to a benign polymorphism that may be G, A, or C. Deletion probes, rather than universal base or unmatched probes, resulted in the greatest T_m separation of mutant and wild type sequences (Figure 5 and data not shown). Although a wild type probe separated all wild type and mutant alleles by 1°C, a deletion in the probe at the polymorphism location increased this separation to 3°C, with one exception (Figure 5). The wild type 'GA' and the mutant 'AG' allelic sequences resulted in very similar T_ms (Figure 5B), because both these alleles had only a single 'A' bulge with the masking deletion probe (Figure 5C).

Discussion

Unlabeled masking probes and high-resolution melting analysis can detect, locate, and genotype a mutation in the background of other mutations or polymorphisms. Masking can be achieved with universal bases, deletions or unmatched nucleotides over the polymorphic region,

so that targeted pathogenic mutations can be clearly identified. The ability of universal bases (DNA base analogues) to increase the tolerance of probes or primers to polymorphic sequences is well known (27,28). The deletion and unmatched nucleotide probes often performed as well as the more expensive universal base probes, as judged by the ΔT_m of wild type and masked alleles. When unmatched nucleotide probes are used for masking, one unmatched nucleotide can be better than the other, as predicted by nearest neighbor thermodynamics of mismatches (29,30).

In some hybridization probe melting assays, wild type probes cannot distinguish all possible sequence variations (11,12,16). If multiple unique alleles are likely and their probe T_ms are similar, definitive genotyping is not possible without further analysis. For example, RET exon 13 contains a polymorphism and a mutation with similar T_m (Figure 1). The masking probes provide unambiguous genotyping of the targeted mutation during melting analysis, preventing the polymorphism from being interpreted as a mutation. Mismatches introduced by the masking probes cause all alleles to shift to a lower T_m, but the ΔT_m between the mutant and wild type alleles are generally increased or unaffected.

Hybridization probe melting assays can also use probes complementary to the targeted mutation to distinguish the mutant allele from other sequence variation (26). In this case, mutant alleles are perfectly matched, while wild type alleles have one mismatch. Single base polymorphisms under the probe, not at the position of the mutation, will result in two mismatches, as long as the mutant allele and the polymorphism are not in *cis*. If the mutation and polymorphism are in *cis*, only one mismatch results and there is a risk of interpreting the mutation as wild type. Another possible failure of mutation-specific probes occurs when mutations result from more than one base change at the same position (e.g. G>A, T or C). If only one mutation-specific probe is used, other mutations at the same nucleotide position will

result in a single mismatch with the probe and could be interpreted as wild type. Masking probes can decrease the number of probes needed for mutation detection and decrease the risk of aberrant results that may be generated with melting analysis using the mutation-specific probe. If masking mismatches are incorporated into mutation-specific probes, the detected mutations can be confirmed while masking other nearby mutations or polymorphisms that would complicate analysis.

Masking probes can be used to analyze one mutation at a time in complicated gene sequences, or to locate the mutation to a codon (as demonstrated for RET exon 10 in Figure 3) or a single nucleotide position (as demonstrated for exon 11 in Figure 4). When mutations in one codon are masked with a three base pair deletion, mutations within the other codon are identified by a lower T_m. Yet, any other sequence variation not at the two pathogenic codons would be clearly identified by an additional mismatch with both masking codon deletion probes. If single base deletion probes are used, the exact nucleotide position of the mutation can usually be identified.

Masking probes that incorporate either a deletion, universal base or unmatched nucleotide can be used when at least one matched base pair is between the sequence variation and the targeted mutation. Aberrant results were sometimes obtained when masking immediately adjacent sequence variation with deletion probes. For example, when the deletion was immediately adjacent to the first position of codon 634 (Figure 4), mutant and wild type alleles had similar stability. Using an unmatched 'T' or 'G' for masking instead of the deletion allowed clear distinction between the mutant and wild type alleles. An additional limitation of deletion probes for masking immediately adjacent sequence variation is illustrated in Figure 5C. Single base deletion probes create a single base bulge in the target DNA strand, usually at the position

of the deletion, but the bulge can be position degenerate depending on nearest neighbors (31). An immediately adjacent mutation would be expected to result in further destabilization (a mismatch next to a single base bulge). However, if the mismatched nucleotide in the probe can complement the otherwise bulged base in the target, then the position of the bulge "shifts", resulting in a single base bulge surrounded by matched pairs. In this case, both the wild type and mutant duplexes have a similar stability (both single base bulges). Such a situation cannot be avoided when the possible nucleotides for the mutation and the adjacent polymorphism are the same (e.g. both G>A). In other cases, this complication can usually be avoided by choosing a probe sequence (wild type or mutant nucleotide) that will not complement the possible adjacent bulged nucleotides. Alternatively, a masking probe with an unmatched nucleotide or universal base at the polymorphism location can be used.

Polymorphism masking should also be useful with other probe designs, including Hybprobes®, Eclipse™ probes, SimpleProbes® and Taqman™ probes (12,14,15,26,27,32,33). Mismatches in probes at non-polymorphic locations have been used previously to increase discrimination between mutant and wild type alleles (26,33,34), indicating that these probes should tolerate mismatches incorporated for masking. The masking probe length may need to be increased if the incorporated mismatches lower the hybridization temperature of the probe to a degree that compromises detection.

Microarrays could also benefit from masking of polymorphisms (35). Masking probes could be designed to tolerate only benign polymorphisms, allowing detection of wild type and mutant alleles. Mismatches have been introduced at non-polymorphic locations in microarray probes to increase probe specificity to its target (36) and a systematic analysis of the hybridization properties of mismatched microarray probes have recently been investigated (37).

These reports suggest that microarray probes should tolerate masking mismatches over selected sequence variation for the specific analysis of neighboring sequence.

Polymorphism masking allows genotyping of targeted mutations within a complicated background of possible sequence variations. Regions that were previously difficult to analyze with probes because of surrounding polymorphisms or multiple possible mutations can now be analyzed using the masking technique. The use of masking probes reduces the need for sequencing in many genotyping assays.

Acknowledgements: We thank Idaho Technology for providing the dye, LCGreen® PLUS. Aspects of high-resolution melting analysis are licensed from the University of Utah to Idaho Technology. Dr. Carl T. Wittwer holds equity interest in Idaho Technology. We thank Dr. Michael Liew for the HPA6 templates. We thank Dr. Karl Voelkerding and Maria Erali for helpful discussions and reviewing the manuscript.

Figure legends:

Figure 1: Masking a polymorphism near the targeted mutation. (A) The diagram illustrates RET exon 13 with the pathogenic codon 768 in red and the polymorphism codon 769 in blue. The '**X**' represents the location of the sequence variation within the codons. The codon 769 (CTT>CTG) polymorphism sequence for the complementary strand is displayed (A>C). The masking probes have wild type sequence with an incorporated mismatch of a universal base, unmatched nucleotide or deletion at the polymorphism location, represented by the '**V**'. The graphs (B-F) are derivative plots of high-resolution melting analysis data using unlabeled probes. For each graph: the black curve is homozygous wild type, the red curve is a heterozygous mutation 768 (GAG>GA**C**), the dark blue curve is a heterozygous codon 769 polymorphism and the light blue curve is a homozygous codon 769 polymorphism. Two derivative melting temperature ranges are underlined on each panel, listing which alleles melted at each T_m range. (B) The wild type probe (WT exon 13 probe) was used over codons 768 and 769. Heterozygous samples have two derivative melting peaks while homozygous samples have only one peak. The codon 769 polymorphism was masked by four different masking probes: (C) deletion probe (D) universal base probe (5'-nitroindole), and (E) unmatched nucleotide 'A' or (F) unmatched nucleotide 'C' probe.

Figure 2: Masking a polymorphism between two pathogenic codons. (A) The diagram illustrates RET exon 11 with pathogenic codons 630 and 634 shown in red. Codon 631 is blue with an '**X**' to represent the location of polymorphism. The masking probes have wild type sequence that incorporates either a deletion or an unmatched nucleotide at the polymorphism

location, represented by the 'V'. The graphs (B-E) are derivative plots of high-resolution melting analysis data using unlabeled probes. For each graph: the black curve is homozygous wild type, the red curves are three unique heterozygous mutations at codon 634(TGC>**G**GC, **C**GC and T**C**C), and the blue curve is a heterozygous polymorphism at codon 631(GAC>GA<u>T</u>). Two derivative melting temperature ranges are underlined on each graph panel, listing which alleles melted at each T_m range. (B) The wild type probe (WT exon 11 probe), over codons 630, 631 and 634 was used for the mutation detection assay. The codon 631 polymorphism was masked by three different masking probes: (C) deletion probe (D) unmatched nucleotide 'T' or (E) unmatched nucleotide 'C' probe.

Figure 3: Analyzing two pathogenic codons that are three nucleotides apart with a masking deletion probe. (A) The diagram illustrates RET exon 10 where pathogenic mutations can be any nucleotide change within codons 609, 611, 618 and 620; all of wild type sequence TGC. Codons 609 and 618 are shown in blue, while codons 611 and 620 are red. Each masking probe has a three base pair deletion of the wild type probe sequence over one pathogenic codon. For the graphs (B-G): heterozygous mutations at codons 609 and 618 are the blue traces, heterozygous mutations at codons 611 and 620 are the red traces and the black traces are homozygous wild type samples. Codons 609/611 data are displayed in the left panels and codons 618/620 data are displayed in the right panels. Two derivative melting temperature ranges are underlined for each graph with codon mutant alleles (MUT), wild type alleles (WT) and masked codon mutant alleles (MASK) noted in each panel. (B) The wild type probe (WT 609/611 probe), over the codons 609 and 611, was used for the mutation detection assay. (C) The masking 609 deletion probe. (D) The masking 611 deletion probe. (E) The wild type probe

(WT 618/620 probe), over the codons 618 and 620, was used for the mutation detection assay. (F) The masking 618 deletion probe. (G) The masking 620 deletion probe. The RET exon 10 heterozygous mutations tested were 609(TGC>T**A**C and T**C**C), 611(TGC>T**A**C, **C**GC, and T**T**C), 618(TGC>T**T**C, T**A**C, **G**GC, **C**GC, and T**C**C), and 620(TGC>**A**GC, T**A**C, TG**G**, T**T**C, and T**C**C).

Figure 4: Masking probes with deletions near or adjacent to the targeted mutation.

(A) The diagram illustrates RET exon 11 where pathogenic mutations can be any nucleotide change within codon 634 (boxed) of the wild type sequence TGC. Each masking probe incorporates a one base pair deletion in the wild type sequence, near or within codon 634 as illustrated in the diagram. Mutations are listed for each position of codon 634 and the melting curves are color coded by mutation position. For each graph (B-G): red traces are unique heterozygous mutations at the first position of codon 634 (red mutations), dark blue traces are unique heterozygous mutations at the second position of codon 634 (blue mutations), while light blue traces are the heterozygous mutation at the third position of codon 634 (light blue mutations). The black traces are homozygous wild type samples. (B) Wild type probe (WT 634 probe). (C-G) Masking deletion probes 1 through 5. Mutations that should be masked by a deletion probe are noted in the panels by the word '**Mask**' in the mutation color. The RET exon 11 mutations tested were 634 (TGC> **A**GC, **C**GC, **G**GC, T**A**C, T**C**C, T**T**C, and TG**G**).

Figure 5: Masking immediately adjacent sequence variation with deletion probes.

Homozygous engineered templates of six different combinations of the G>A mutation and adjacent polymorphism sequences were tested: GA, GC, GG, AA, AC, and AG. The mutant 'A'

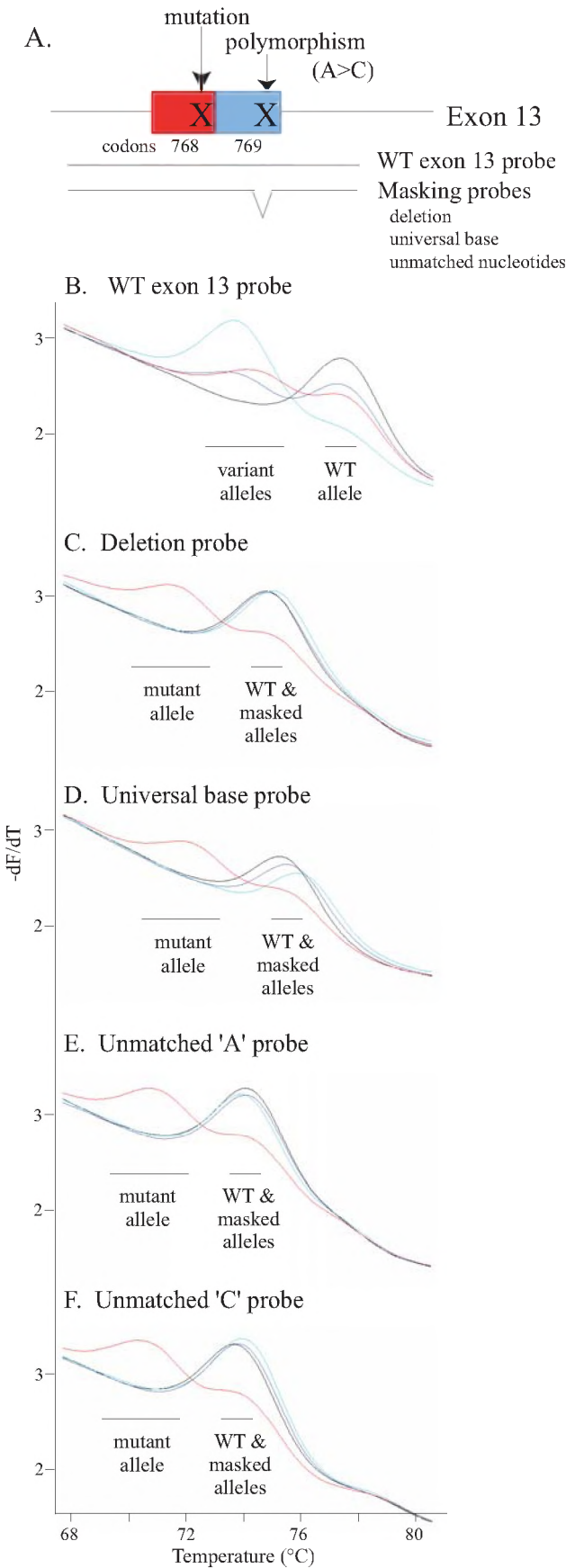
sample traces are in red, while the wild type 'G' sample traces are in blue. (A) The wild type probe (WT HPA6 probe). (B) Masking deletion probe. The mutant 'AG' allele with a T_m suggesting wild type is labeled in bold red with a thick trace line. (C) The proposed duplexes of the two genotypes with very similar T_ms are displayed. The target sequences are shown above the complementary masking deletion probe sequence, with the mutation location (MUT) and polymorphism location (POLY) indicated. The dash '- ' indicates the position of the deletion, located opposite the unpaired, bulged nucleotide. For the mutant 'AG' allele with the masking deletion probe, the expected duplex with a mismatch and a single bulged nucleotide at the polymorphism position is displayed above the actual duplex with only a bulged nucleotide at the mutant position. For both the wildtype allele and mutant 'AG' allele, the single base bulge 'A' is surrounded by matched base pairs to result in similar T_ms.

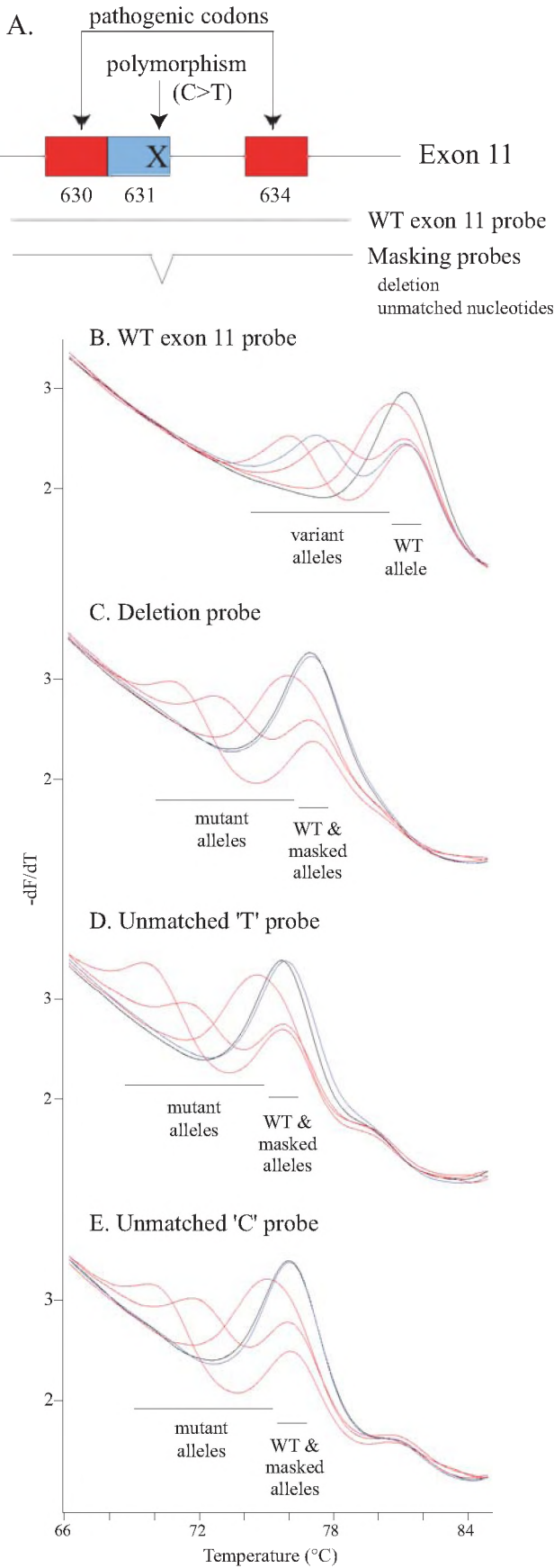
References:

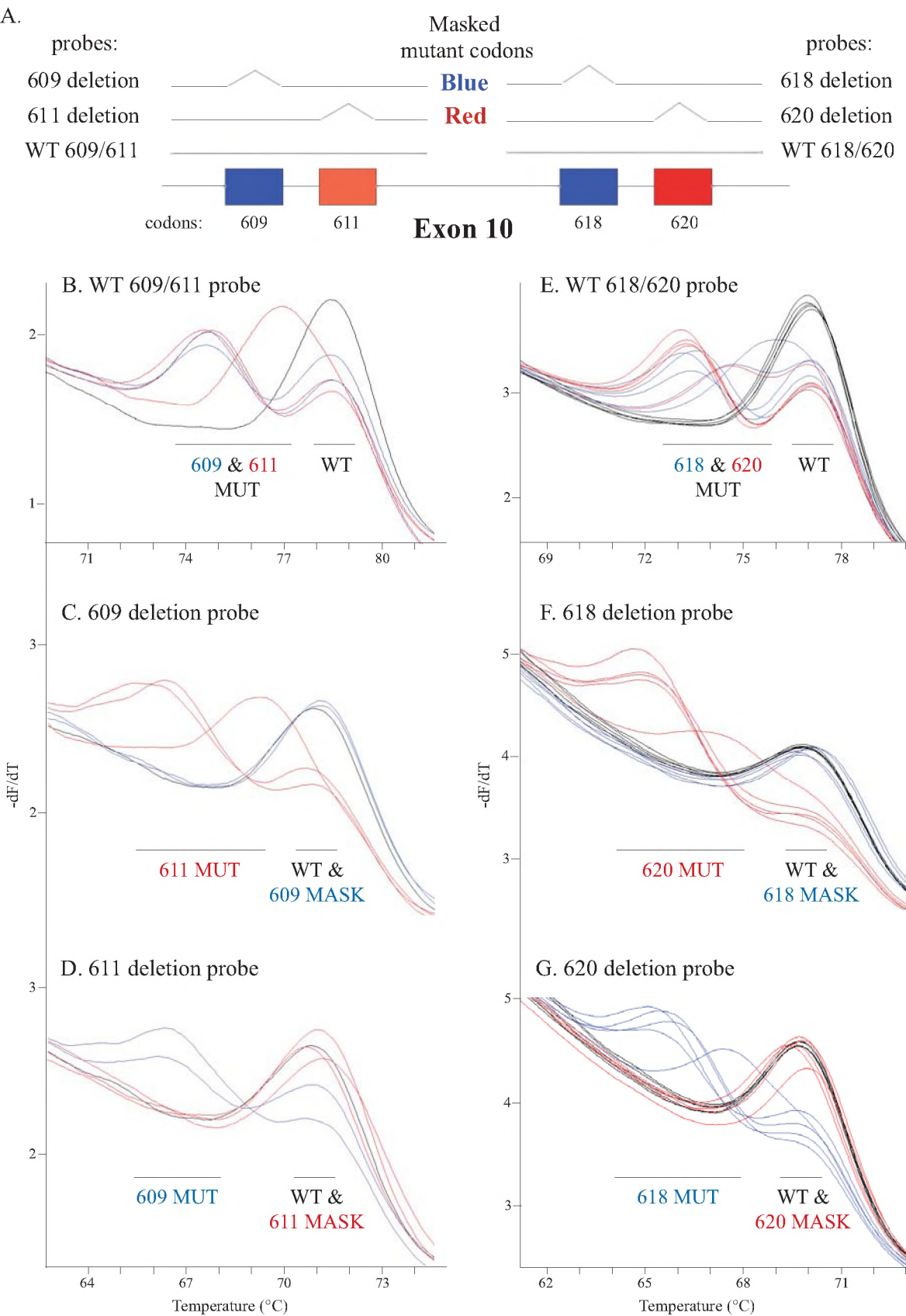
1. Kim, I.J., Kang, H.C., Park, J.H., Ku, J.L., Lee, J.S., Kwon, H.J., Yoon, K.A., Heo, S.C., Yang, H.Y., Cho, B.Y. *et al.* (2002) *Clin Cancer Res*, 8, 457-463.
2. Kruckeberg, K.E. and Thibodeau, S.N. (2004) *Clin Chem*, 50, 522-529.
3. de la Fuente, M., Quinteiro, C., Dominguez, F. and Loidi, L. (2001) *Clin Chem*, 47, 1131-1132.
4. Ruiz, A., Antinolo, G., Marcos, I. and Borrego, S. (2001) *Clin Chem*, 47, 1939-1944.
5. Xue, F., Yu, H., Maurer, L.H., Memoli, V.A., Nutile-McMenemy, N., Schuster, M.K., Bowden, D.W., Mao, J. and Noll, W.W. (1994) *Hum Mol Genet*, 3, 635-638.
6. Bugalho, M.J., Domingues, R. and Sobrinho, L. (2002) *BMC Genet*, 3, 8.
7. Roque, M., Pusiol, E., Perinetti, H., Godoy, C.P. and Mayorga, L.S. (2002) *BMC Med Genet*, 3, 4.
8. Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G. and Pryor, R.J. (2003) *Clin Chem*, 49, 853-860.
9. Gundry, C.N., Vandersteen, J.G., Reed, G.H., Pryor, R.J., Chen, J. and Wittwer, C.T. (2003) *Clin Chem*, 49, 396-406.
10. Liew, M., Pryor, R., Palais, R., Meadows, C., Erali, M., Lyon, E. and Wittwer, C. (2004) *Clin Chem*, 50, 1156-1164.
11. Zhou, L., Myers, A.N., Vandersteen, J.G., Wang, L. and Wittwer, C.T. (2004) *Clin Chem*, 50, 1328-1335.
12. Warshawsky, I., Hren, C., Sercia, L., Shadrach, B., Deitcher, S.R., Newton, E. and Kottke-Marchant, K. (2002) *Diagn Mol Pathol*, 11, 152-156.
13. von Ahsen, N., Oellerich, M., Armstrong, V.W. and Schutz, E. (1999) *Clin Chem*, 45, 2094-2101.
14. Schutz, E., von Ahsen, N. and Oellerich, M. (2000) *Clin Chem*, 46, 1728-1737.
15. Lyon, E., Millson, A., Phan, T. and Wittwer, C.T. (1998) *Mol Diagn*, 3, 203-209.
16. von Ahsen, N., Schutz, E., Armstrong, V.W. and Oellerich, M. (1999) *Clin Chem*, 45, 694-696.
17. Collins, F.S., Brooks, L.D. and Chakravarti, A. (1998) *Genome Res*, 8, 1229-1231.
18. Orban, T.I., Csokay, B. and Olah, E. (2000) *Biotechniques*, 29, 94-98.
19. Eng, C. and Mulligan, L.M. (1997) *Hum Mutat*, 9, 97-109.
20. Stenson, P.D., Ball, E.V., Mort, M., Phillips, A.D., Shiel, J.A., Thomas, N.S., Abeysinghe, S., Krawczak, M. and Cooper, D.N. (2003) *Hum Mutat*, 21, 577-581.
21. Wang, R., McFarland, J.G., Kekomaki, R. and Newman, P.J. (1993) *Blood*, 82, 3386-3391.
22. Ceccherini, I., Hofstra, R.M., Luo, Y., Stulp, R.P., Barone, V., Stelwagen, T., Bocciardi, R., Nijveen, H., Bolino, A., Seri, M. *et al.* (1994) *Oncogene*, 9, 3025-3029.
23. Elisei, R., Cosci, B., Romei, C., Bottici, V., Sculli, M., Lari, R., Barale, R., Pacini, F. and Pinchera, A. (2004) *J Clin Endocrinol Metab*, 89, 3579-3584.
24. Barik, S. (1998) In Horton, R. M. and Tait, R. C. (eds.), *Genetic Engineering with PCR.* Horizon Scientific Press, Wymondham, UK, pp. 25-37.
25. Primer3. ((accessed 2004)).

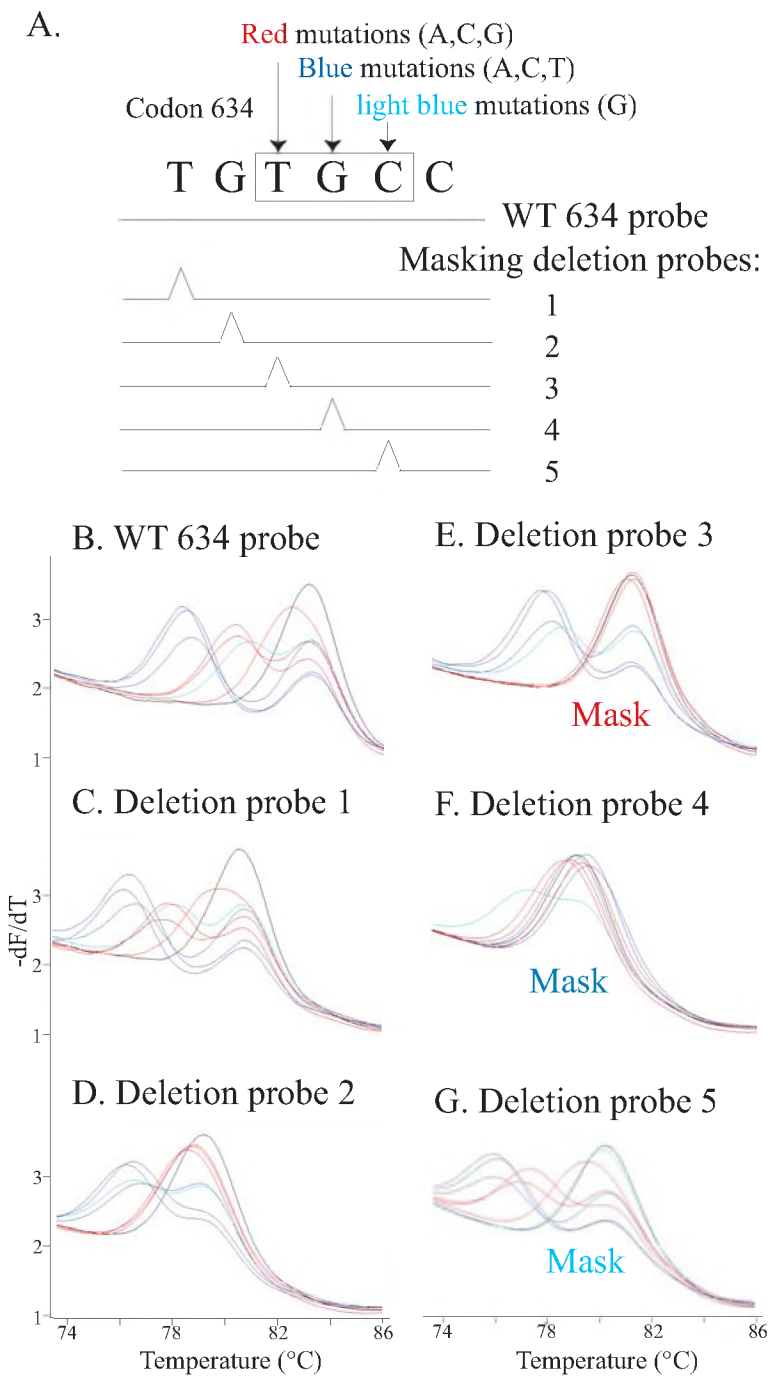
26. **Lyon, E. (2001) *Expert Rev Mol Diagn*, 1, 92-101.**

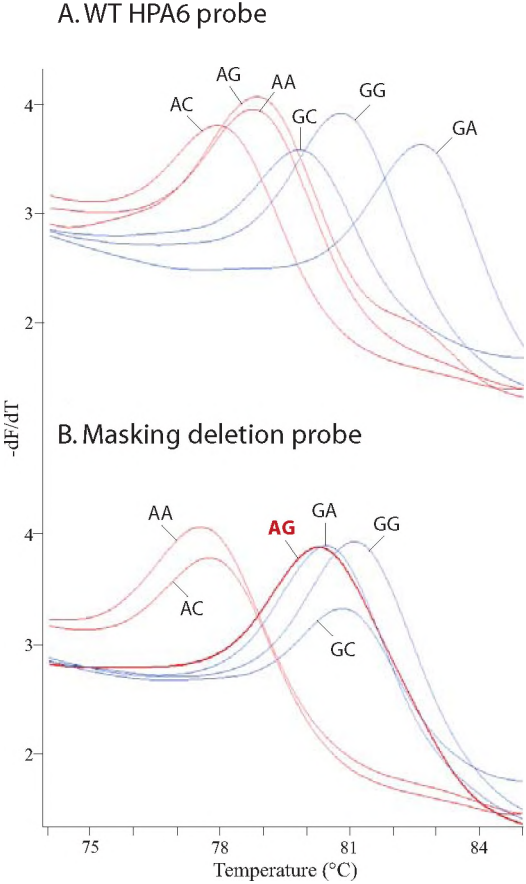
27. **Szemes, M. and Schoen, C.D. (2003) *Anal Biochem*, 315, 189-201.**
28. **Loakes, D. (2001) *Nucleic Acids Res*, 29, 2437-2447.**
29. **Peyret, N., Seneviratne, P.A., Allawi, H.T. and SantaLucia, J., Jr. (1999) *Biochemistry*, 38, 3468-3477.**
30. **Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A. (1986) *Proc Natl Acad Sci U S A*, 83, 3746-3750.**
31. **Zhu, J. and Wartell, R.M. (1999) *Biochemistry*, 38, 15986-15993.**
32. **Bernard, P.S., Ajioka, R.S., Kushner, J.P. and Wittwer, C.T. (1998) *Am J Pathol*, 153, 1055-1061.**
33. **Teupser, D., Rupprecht, W., Lohse, P. and Thiery, J. (2001) *Clin Chem*, 47, 852-857.**
34. **Guo, Z., Liu, Q. and Smith, L.M. (1997) *Nat Biotechnol*, 15, 331-335.**
35. **Erali, M., Schmidt, B., Lyon, E. and Wittwer, C. (2003) *Clin Chem*, 49, 732-739.**
36. **Lee, I., Dombkowski, A.A. and Athey, B.D. (2004) *Nucleic Acids Res*, 32, 681-690.**
37. **Karaman, M.W., Groshen, S., Lee, C.C., Pike, B.L. and Hacia, J.G. (2005) *Nucleic Acids Res*, 33, e33.**


A.
mutation
polymorphism
(A>C)
X
X
Exon 13
codons 768 769
WT exon 13 probe
Masking probes
deletion
universal base
unmatched nucleotides
B. WT exon 13 probe
variant alleles
WT allele
C. Deletion probe
mutant allele
WT & masked alleles
D. Universal base probe
mutant allele
WT & masked alleles
E. Unmatched 'A' probe
mutant allele
WT & masked alleles
F. Unmatched 'C' probe
mutant allele
WT & masked alleles
-dF/dT
3
2
68
72
76
80
Temperature (°C)


A.
pathogenic codons
polymorphism
(C>T)
X
Exon 11
630
631
634
WT exon 11 probe
Masking probes
deletion
unmatched nucleotides
B. WT exon 11 probe
variant alleles
WT allele
C. Deletion probe
mutant alleles
WT & masked alleles
D. Unmatched 'T' probe
mutant alleles
WT & masked alleles
E. Unmatched 'C' probe
mutant alleles
WT & masked alleles
-dF/dT
3
2
66
72
78
84
Temperature (°C)


A.
probes:
609 deletion
611 deletion
WT 609/611
Masked
mutant codons
Blue
Red
probes:
618 deletion
620 deletion
WT 618/620
codons:
609
611
Exon 10
618
620
B. WT 609/611 probe
609 & 611
MUT
WT
E. WT 618/620 probe
618 & 620
MUT
WT
C. 609 deletion probe
611 MUT
WT &
609 MASK
F. 618 deletion probe
620 MUT
WT &
618 MASK
D. 611 deletion probe
609 MUT
WT &
611 MASK
G. 620 deletion probe
618 MUT
WT &
620 MASK
-dF/dT
Temperature (°C)
Temperature (°C)


A.
Red mutations (A,C,G)
Blue mutations (A,C,T)
light blue mutations (G)
Codon 634
T G T G C C
WT 634 probe
Masking deletion probes:
1
2
3
4
5
B. WT 634 probe
E. Deletion probe 3
Mask
C. Deletion probe 1
F. Deletion probe 4
Mask
D. Deletion probe 2
G. Deletion probe 5
Mask
-dF/dT
Temperature (°C)
Temperature (°C)



C.

WT 'GA' allele

GA target cc**G** ga
Masking probe gg**C** - ct

MUT POLY

Mutant 'AG' allele

Expected: Mismatch + Bulge

AG target cc**A** ga
Masking probe gg**C** - ct

Actual: Bulge only

AG target cc **G**ga
Masking probe gg - **C**ct

MUT POLY

Table 1: Primers and Probes

RET exon	Primers[a]	Amplicon (base pair)	Codon of variation[b]	Probes (base pairs)[c]	Probe sequence[d]
10	GGGCAGCATTGTTGGGGGAC	146	609,611	WT 609/611 (30bp)	GGCTATGGCACC**TGC**AAC**TGC**TTCCCTGAG
	TGGTGGTCCCGGCCGCCA		618,620	WT 618/620 (31bp)	GGAGAAG**TGC**TTC**TGC**GAGCCCGAAGACATC
11	TGCCAAGCCTCACACCAC	109	630, 631	WT exon 11 (27bp)	CGTGCG**GCA**CAGCTCGTC**GCA**CAGTGG
	GACAGCAGCACCGAGAC		634	WT 634 (31bp)	TGCGATCACCGTGCG**GCA**CAGCTCGTC**GCA**C
13	ACTTGGGCAAGGCGATGCAG	274	768,769	WT exon 13 (30bp)	CCCGAGTGA**G**CTTCGAGACCTGCTGTCAGA
	GAACAGGGCTGTATGGAGC				
HPA6	TGGGATCCCAGTGTGAGTGCTCA	180		WT HPA6 (31bp)	CTGCAGACGGGCTGACCCTCT**C**GGGGGCTGC
	AGAAGTCGTCACACTCGCAGTAC				

[a] Primers are listed 5' to 3', with the forward primer above the reverse primer.
[b] The underlined codons contain a polymorphism, while the other codons contain pathogenic mutations.
[c] WT - wild type
[d] Probe sequences are wild type and listed 5' to 3'. RET exon 10 and 13 are forward probes, while RET exon 11 and HPA6 gene are reverse probes. The possible mutant locations are highlighted in **bold** and the polymorphism locations are underlined. The masking probes have the same sequence as the wild type probes, except at the incorporated masking mismatch(es) displayed in each figure. The universal base and unmatched nucleotide masking probes were the same size (base pair) as the wild type probes, while the masking deletion probes were reduced in size by the number of deleted nucleotides from the wild type probe sequence.

Highsmith, William E., Ph.D.
From: Highsmith, W. Edward Jr., Ph.D. [Highsmith.W@mayo.edu]
Sent: Monday, March 22, 2004 3:42 PM
To: IRB Minimal Risk Protocol; Highsmith, W. Edward Jr., Ph.D.; Biospecimens Committee
Cc: Highsmith, W. Edward Jr., Ph.D.
Subject: Request for Minimal Risk Protocol Approval

Minimal Risk Protocol Summary

This form will be submitted simultaneously to both the Institutional Review Board and the Biospecimens Subcommittee at Rochester or Scottsdale (if needed). In general, review and approval by both bodies is required prior to activation of the study.
Questions concerning the role of the Institutional Review Board should be directed to: Cindy L. Boyer, Research Services, 6-2808
Questions concerning the role of the Rochester Biospecimens Subcommittee should be directed to: Cheryl Nelson, Rochester Research Services, 4-5920
Questions concerning biospecimens in Scottsdale should be directed to: Linda Romme, Scottsdale Research Services, 2-4443.
Questions or comments regarding this form should be directed to the IRB Office.

LIVING OR DECEASED1 both
BIOSPECIMENS1 YES
DATATYPE1 deidentified
EXTERNAL COLLABORATORS1 YES
INTEND TO PUBLISH YES
PROPOSAL TITLE Provision of de-identified samples to ARUP laboratories for method validation
SITE ROC
PRINCIPAL INVESTIGATOR Highsmith, W. Edward Jr., Ph.D.
PI ID 14143372
CO INVESTIGATOR1 NotAnswered
CO INVESTIGATOR2 NotAnswered
CO INVESTIGATOR3 NotAnswered
CO INVESTIGATOR4 NotAnswered
CO INVESTIGATOR5 NotAnswered
STUDY COORDINATOR NotAnswered
SC ID NotAnswered

PROJECT PROPOSAL Melt-curve analysis is a newly developed technology

for the high-throughput, inexpensive detection of mutations in PCR amplified DNA. Dr. Rong Mao, a former fellow in the Mayo Molecular Genetics Laboratory (MGL), and colleagues at the University of Utah and ARUP Laboratories have developed a melt-curve analysis platform for the detection of mutations in the RET protooncogene using the HR1 High Resolution Melter from Idaho Technologies. Their work parallels work that is currently being done in the Mayo MGL using the same instrument. I propose to send Dr. Mao up to 60 de-identified samples that have been previously characterized with respect to RET gene mutations as part of clinical evaluations for the inherited cancer syndrome multiple endocrine neoplasia, type 2A. These samples either have been or will be evaluated on the HR1 platform in the Mayo MGL in an ongoing study exempted by the IRB April 1, 2003. We will collaborate on optimization and validation of an assay whcih could be faster and less expensive than currently existing methods.
FUNDING SOURCE n/a
FUND AMOUNT n/a

METHODS 1-2 examples of the approximately 40 disease causing RET mutations identifed by the Mayo MGL will be de-idetified and sent to Dr. Mao at ARUP Laboratories. No patient indentifiers will be included. The only information to accompany the specimen will be the identity of the RET mutation.
DATA OR SPECIMENS SOURCE Existing biospecimen
OTHER DATA OR SPECIMENS SOURCE NotAnswered
GCRC USEAGE No
COLLABORATOR NAMES Dr. Rong Mao
COLLABORATING INSTITUTIONS ARUP [ARUP is a commercial reference laboratory owned and operated by the University of Utah]
ACADEMIC INSTITUTION Yes
COMMERCIAL INSTITUTION Yes
BIOSPECIMENS OUTSIDE MAYO Yes
CONTACT INFORMATION Rong Mao, MD Associate Medical Director Molecular Genetics Section ARUP Laboratories Adjunct Assistant Professor of Pathology University of Utah School of Medicine Chipeta Way Salt Lake City, UT 84108 Tel: 801-583-2787 x 3165 Fax: 801-584-5207 e-mail: rong.mao@aruplab.com
EXTERNAL COLLABORATOR ROLE Evaluation of the HR1 method for mutation identification in the RET gene.
CLINICAL MATERIAL TO EXTERNAL COLLABORATORS De-identified DNA
BIOSPECIMEN TYPE DNA
BIOSPECIMEN SOURCE DNA
BIOSPECIMEN OTHER SOURCE NotAnswered
BIOSPECIMENS COLLECTED Existing
BIOSPECIMEN SAMPLE NUMBER 60
BIOSPECIMEN IDENTIFICATION Other
OTHER ID Mutation previously identified in clinical test
SPECIMENS STORAGE BUILDING Hilton

SPECIMENS STORAGE FLOOR 9
SPECIMENS STORAGE ROOM 9-16
SPECIMENS STORAGE OTHER NotAnswered
BIOSPECIMEN GERMLINE TESTING YES
RESULTS TO PATIENT OR RECORD NO
DE IDENTIFIED DATA No
SURVEY RESEARCH NO
ROCHESTER EPIDEMIOLOGY USED NO
NON MAYO PATIENT INFO NO
RESIDENTS OLMSTED COUNTY NO
PARTICIPANT CONTACT NO
HIPAA WAIVER CONFIDENTIAL DATA Yes
HIPAA WAIVER SUBJECT IDENTIFIERS DESTROYED Yes
HIPAA WAIVER SUBJECT IDENTIFIERS Yes
HIPAA WAIVER IDENTIFICATION Yes
WAIVER CONSENT MINIMAL RISK Yes
WAIVER CONSENT NO ADVERSE EFFECT SUBJECT Yes
WAIVER CONSENT REQUIRED TO DO RESEARCH Yes
WAIVER CONSENT SUBJECTS ADDITIONAL INFORMATION Yes
REQ EMAIL Highsmith.W@mayo.edu

Emailed to:
irbminimalriskprotocol@mayo.edu,Highsmith.W@mayo.edu,biospecimens@mayo.edu
PI email: Highsmith.W@mayo.edu

MAYO FOUNDATION

Please direct any questions regarding the completion of this form to the IRB Office at the phone extension or e-mail address to the left.

FROM: MAYO FOUNDATION INSTITUTIONAL REVIEW BOARDS
201 BUILDING, ROOM 4-60
PHONE 4-2329 • FAX 8-0051 • E-MAIL irbprogressreports@mayo.edu

DATE: **02/28/2005**

TO: **HIGHSMITH,W,E Jr., PhD**

REVIEW TYPE: **Expedited**

RE: ANNUAL REVIEW OF IRB PROTOCOL **701-04** REVIEW COMMITTEE: **Expedited Review Committee**
"Provision of De-identified Samples to ARUP Laboratories for Method Validation"

Progress Report Instructions and Report Form

Please read these instructions completely and carefully

According to our records, the IRB has previously sent a progress report reminder notification. Federal regulation [45CFR46.1009(e)] requires the Institutional Review Boards (IRB) to review protocols at intervals appropriate to the degree of risk, but not less than once per year. At this time, the due date for the above named protocol's annual review is now 30 days away. **Approval of this protocol will expire on Mar-29-2005 unless the IRB approves a completed progress report prior to this date.** You are responsible for submitting a continuing or final progress report with all required materials in time for review by the IRB before this expiration date. *Failure to submit a complete progress report may cause your protocol to expire before it can be approved.* Please note that the deadline to make an Expedited Review Committee agenda, the deadline is noon central time, the Thursday prior to the meeting. Note that the deadline for an agenda may change due to holidays.

A complete progress report ***must*** include a **single-sided** copy of the most recently IRB-approved consent form(s) (if applicable). Double-sided copies will **not** be accepted. This document does not need to be included if the answer to 3a is "Yes" and the number entered for question 4 is "0" (zero).

DO NOT include registration numbers (clinic numbers) or any other patient identifiers in your progress report submission.

If all supporting documents to the progress report can be sent electronically, please e-mail the documents (along with this completed form) as separate attachments in the same e-mail, using "Progress Report" for the subject, to irbprogressreports@mayo.edu. Do not combine the progress report form with other materials into a singular attachment for e-mail. Submissions of this kind will not be accepted by the IRB.

If **any** of the supporting documents cannot be sent electronically, please print this completed progress report form, place it on top of the packet of the supporting documents, and send the entire packet to: IRB Progress Reports Secretary, 201 Building, Room 460.

Please do not submit more than one copy of your completed progress report to the IRB.
Keep a copy of your entire progress report for your records!

If the protocol involves the General Clinical Research Center (GCRC), you are responsible for sending a complete copy of the progress report and all supporting materials (except the protocol) to **Shari Brumm, GCRC, Domitilla 5-521**

Progress Report Form

Date: **02/28/2005**

Name of Principal Investigator: **HIGHSMITH,W,E Jr., PhD**

Review Type: **Expedited**

IRB #: **701-04**

Review Committee: **Expedited Review Committee**

Title: **"Provision of De-identified Samples to ARUP Laboratories for Method Validation"**

Expires: **Mar-29-2005**

If the IRB consent type for this protocol is "waived," please complete the online progress report form at http://wolfpack2.mayo.edu/resis/irb/chart_review.cfm instead of using this form. The address above will need to be typed into your web browser's address bar.

Please complete this form by clicking on the appropriate check boxes and typing in the text fields.

PLEASE TYPE ALL NARRATIVE COMMENTS

Conflicts of Interest: The following reflects the current status for all study personnel:

☒ There are no new conflicts to disclose

☐ One or more study personnel now have a conflict of interest. (Please contact the Conflict of Interest (COI) Review Board to report and resolve this conflict before submitting to the IRB. A copy of the minute item response from the COI Review Board should be forwarded with this submission).

Please answer the following question BEFORE continuing with the rest of this form.

Does this IRB number refer to a grant application under which all active protocols are separately submitted to the IRB for review (i.e., no subjects are enrolled or no patient data collected under this IRB number)? .....Yes ☐ No ☒

→ **If "Yes", please list the IRB numbers (or titles if an IRB number has not yet been assigned) of protocols supported by this grant in the box below and then answer only questions 1 and 2.**

COMPLETION OF THIS SECTION IS REQUIRED FOR ALL STUDIES WHERE HIPAA AUTHORIZATION IS NOT BEING OBTAINED

Request for Waiver of HIPAA Authorization

A Request for Waiver of HIPAA Authorization is required in accordance with 45 CFR 164.512(i).

Please complete this section by checking all boxes that apply.

☒ All study data will be treated in a confidential manner and the same precautions used to protect patient clinical data will be employed.
NOTE: *If you are unable to check this box, please describe in the box below the precautions that will be taken to prevent inappropriate use of the data.*

☒ All subject identifiers will be destroyed upon completion of the research.
NOTE: *If you are unable to check this box, please explain in the box below why the retention of the identifiers is appropriate.*

☒ I certify that the subject identifiers will not be reused or disclosed to any other person or entity, except as required by law, for authorized conduct and oversight of the study, or for other IRB-approved research.

☒ The research could not be practicably carried out without access to and use of the subjects' identifying information.

I. Protocol Status

1. **Do you want to continue this protocol in an active status? (If any participants are still receiving study intervention or are being followed per protocol, the protocol must continue in an active status.) .....Yes ☐ No ☒**

2. This protocol is being conducted under this IRB number at (check all that apply) .....MCR ☐ MCJ ☐ MCS ☐
If this protocol Is being conducted at more than one Mayo site under this IRB number, It Is the responsibility of the protocol's principal Investigator to submit a progress report that includes data from all participating Mayo sites.

II. Protocol Activity

3a. Is the research permanently closed to the enrollment of new people? ..... Yes ☒ No ☐

3b. If "Yes", have all currently enrolled participants completed study interventions? ..... Yes ☒ No ☐

4. How many participants have been enrolled at Mayo since IRB approval was last received? **50**
If this is the first progress report for this protocol, please enter the same number in questions 4 and 5. Do not leave either field blank.

5. How many participants have been enrolled at Mayo since the study was originally approved? **50**

6. How many participants (at Mayo) have been approved for enrollment by the IRB? **60**

7. If the IRB approved screening of additional participants in order to meet target accrual, please indicate the total number approved for screening (that is, the total number approved for enrollment plus additional screens) **60**
If there is no approval of additional participants for the purposes of screening, please enter the response from question 6 in the box for question 7. Do not leave either field blank.

8. Are Mayo participants still being followed per protocol? ..... Yes ☐ No ☒

9. Briefly summarize (in the box below, in 200 words or less) the protocol activity since IRB approval was last received. Include progress to date and future plans.
50 de-identified samples with previously characterized RET protooncogene mutations were sent to Dr. Mao at ARUP Laboratories for validation of a new test protocol. Results have been presented as a poster at a national meeting.

10a. Have any changes occurred to the Mayo personnel involved with this study that have not been submitted to the IRB via the Protocol Modification Request Form? ..... Yes ☐ No ☒

10b. If "Yes", please list in the box below the full name and role (i.e., principal investigator, co-investigator, study coordinator, etc.) of all Mayo personnel being added or removed from the study. If any personnel are being replaced, please indicate if they will be remaining on the study under a different role.

Remember that personnel must successfully complete the Mayo Training Program for Protecting Human Subjects (http://researchweb.mayo.edu/mtp-phs/) prior to participating in a human research project.

11a. Have any changes in the specific aims, study procedures, or consent form occurred that have not been approved by the IRB? ..... Yes ☐ No ☒

11b. If "Yes", please explain in the box below.

12a. Have any changes in the eligibility criteria occurred that have not been approved by the IRB? ..... Yes ☐ No ☒

12b. If "Yes", please explain in the box below.

13. Since IRB approval was last received, has the study been audited or monitored by any outside sources (i.e., study sponsor, ECOG, NCCTG, NCI, etc.)? ..... Yes ☐ No ☒
If "Yes", a copy of the sources' audit report, monitor report or summary must be included with this progress report.

14a. Has anything appeared in the pertinent medical literature that affects the conduct of this study, the anticipated benefits, or the potential risks? ..... Yes ☐ No ☒

14b. If "Yes", please explain in the box below.

15. If any publications or presentations have resulted from the work related to this study, please list them in the box below.
Abstract - 1.Margarf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT, Mutation scanning of the RET protooncogene using unlabeled probes and high-resolution melting analysis. J Molec Diag 2004; 6(4):435.

III. Review of Risks to Research Participants

16a. Have any additional risks been identified since IRB approval was last received? ..... Yes ☐ No ☒

16b. If "Yes", and these risks have not been reported to the IRB, please summarize in the box below.

17a. Briefly describe (in the box below) the frequency and severity of all adverse events (including those already reported to the IRB) that have occurred since IRB approval was last received.

None

The investigator is reminded that all serious adverse events must be reported to the Serious Adverse Events/Deviations Board. Do not attach SAE/Deviation forms to this progress report.

17b. Also indicate (in the box below) whether the adverse events are similar in type, frequency and severity to what was expected before the study, and if not, how they differ from expectations.

17c. If this protocol is a multi-center study, please also describe (in the box below) whether Mayo's experience with adverse events in this study is comparable with that at other institutions.

18a. Was there any unusual increase in the frequency of serious but expected adverse events among Mayo participants? ..... Yes ☐ No ☐

18b. If "Yes", please describe in the box below.

IV. Informed Consent Evaluation – (Applies to both written and verbal consent)

19a. Have any problems occurred with regard to obtaining and documenting of the informed consent? ..... Yes ☐ No ☐

19b. If "Yes", please describe in the box below.

20. In the box below, briefly state each reason for the withdrawal of research participants (whether voluntary or not) from the study. For each reason given, please state the number of research participants withdrawn since IRB approval was last received.

21a. Have there been any unanticipated problems with the retention of participants? ..... Yes ☐ No ☐

21b. If "Yes", please describe in the box below.

22. Are the consent/assent form documents still acceptable (i.e., the information contained in the document is accurate and complete and there is no new information that may have been obtained since the last IRB approval which should be disclosed to participants)? .... Yes ☐ No ☐ Verbal Consent ☐

If "No", please e-mail (to irbprogressreports@mayo.edu) an electronic copy of all recommended changes to the consent/assent form(s).